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Theoretical conformational analysis of a family of α-helical immunocytokines

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According to the results of the theoretical conformation analysis and available experimental data, the known immunocytokines can be divided into two groups: α-helical (IFNs-α, β, γ, ω; IL-2, 3, 4, 5, 6, 7; G-, M-, GM-CSFs; cMGF, PDGF) and β-pleated proteins (ILs-1α, β; TNFS-α, β). IFNs-α, β, γ, ω, IL-6, G-CSF, cMGF were shown to form a family of α-helical globular proteins characterized by a statistically significant homology in amino acid sequences and by common features of the secondary structure formation. Comparison of the sequences of 72 IFNs-α, β, ω reveals three clusters of conservative amino acid positions. Their participation in the formation of active sites of IFN-α, β, ω is supposed.

Introduction

Experimental data provide evidence that the amino acid sequences of functionally related proteins (cytochromes, myoglobins, hemoglobins, lysozymes, etc.) vary in evolutionary development much more rapidly than their three-dimensional structure. Therefore, the conclusion of a common evolutionary ancestor and possible functional relationship of distantly related proteins ought to be derived first from the secondary structure homology and only then from the homology of amino acid sequences [1] which could be absent altogether [2]. Previously, on the basis of the secondary structure homology and functional similarity of IFNs-α, β and γ it was concluded that these proteins had a common evolutionary origin [3,4], though the cursory comparison of amino acid sequence of IFN-γ with those of IFNs-α and β did not reveal any statistically valid homology [5]. Using our method of estimation of statistical validity of homology in the structure of hydrophobic cores of high α-helical globular proteins, evidence was obtained that IL-2, IL-3, PDGF and $p28^{sis}$ ought to have the three-dimensional structure similar, in general features, to that of IFNs [6,7]. From these data we suppose that a greater part of the known IFNs, ILs and GFs form a family of proteins united by a structural similarity and common evolutionary origin [7,8].

The cDNA and genes of several new ILs and CSFs have been cloned by now and information on the nucleotide sequence has been obtained which has been translated, respectively, in the amino acid sequence of the proteins coded by them. In a number of cases, a primary structure homology between IFNs (on the one hand) and ILs, CSFs (on the other hand) was revealed (e.g., between IFN-β_2 (BSF-2), G-CSF and cMGF [9,10], and between IL-5 and IFN-γ [11]). Besides, IFN-β_2 displays functional activity of the ILs and is also known as IL-6 [12].

In the present work we aimed at studying, by the methods of theoretical conformational analysis, secondary structures of IFNs, ILs and CSFs in order to obtain additional information on possible structural and evolutionary relationships between them, and on the basis of the analysis of conservative positions and homology in the protein amino acid sequences to localize probable active sites.

Methods

The computer program based on the method of estimation of protein secondary structures from their amino acid composition [13] was used in our work. The

Abbreviations: Hu, human; Mu, murine; IFN, interferon; IL, interleukin; CSF, colony-stimulating factor; BSF, B-cell stimulating factor; PDGF, platelet-derived growth factor; $p28^{sis}$, transforming protein of simian sarcoma virus; PTM, α_1-prothymosin-α_1; cMGF, chicken myelomonocytic growth factor; M, macrophage; G, granulocytic.

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method employs the results of correlation and regression analyses of interrelation between the content of two types of secondary structure (α-helices and β-sheets) and amino acid composition of 80 proteins. The error of percent estimation of α-helices and β-sheets by means of the method does not exceed ± 7 and 10%, respectively, as compared to the data of X-ray analysis obtained for 28 proteins [14].

To predict the localization of secondary structures we used the methods of Zav'yalov [15], Novotny and Auffray [16], Efimov [17], Lim [18], Ptitsyn and Finkelstein [19], Chou and Fasman [20] and Garnier [21]. Statistical validity of primary structure homologies was evaluated according to the method of Finkelstein [22].

Domain boundaries were determined on the basis of the algorithm of Vonderviszt and Simon [23]. The method is based on statistical data on the preference occurrence of amino acid residues at the n and $n+1$, n and $n+2$ positions of the polypeptide chain. Domain boundaries correlate with the minima of the preference profiles.

Results

Table I (columns 3, 4) shows the results of estimation of α-helices and β-sheets for IFNs-α, β, ω and γ, ILs-2, 3, 4, 5, 6, 7, CSFs (M-CSF, G-CSF and GM-CSF) and cMGF. To facilitate the analysis, the data are presented for human proteins (except those for cMGF), though the calculations were carried out for analogous proteins of the other animal species and yielded similar results (the data are not presented). As seen from Table I, the content of α-helices in the proteins under analysis is predicted within the range of 55–75%, whereas the content of β-sheets varies from 0–31%. The experimental data available (columns 5, 6) agree well with the results of estimation from amino acid composition (except for the evaluation of β-sheets in GM-CSF). Thus, the proteins listed in Table I share a common feature, a fairly high extent of α-helicity. They possess except for IL-3, four exons (column 8) in the part of the gene corresponding to the mature protein lacking a leader sequence. For M-CSF, the number of exons is indicated for the N-terminal part of the mature protein formed due to the putative proteolytic cleavage of the membrane-linked precursor [40].

The second logical step of the analysis requires information on localization of secondary structure segments of the polypeptide chain. As an example of analysis of immunocytokine secondary structure by different methods, we present the results obtained for IFN-αA.

Fig. 1 shows the results of prediction of secondary structure and domain borders for IFN-αA by means of different methods [15–21]. All the methods predict a considerable amount of α-helices and insignificant quantity of β-sheets. If one identifies secondary structure sites predicted by all the methods used, this would yield five segments of α-helices and none of β-sheets. All the turns are found in the region between the helical segments. According to the method of Vonderviszt and Simon [23], one can identify two domains in the structure of IFN-αA. Their interface lies at the region of 100–120 residues. Analogous calculations were performed by us for the other types of IFN-α, a number of IFN-β and IFN-ω (the results are not shown). Since

TABLE I

Some features of α-helical proteins, immunocytokines

No.	Protein name	Theoretical determination of content		Experimental determination of content		Quantity of amino acid residues in the mature protein	Quantity of exons in a mature protein gene
		α%	β%	α%	β%		
1	Hu IFN-αA	66	15	45–70 (CD) [24]		165 [29]	
2	Hu IFN-β	63	9	70 (CD) [27]		166 [30]	
3	Hu IFN-γ	64	13	–		146 [31,32]	4 [31,32]
4	Hu IFN-ω_1	68	16	–		172 [33]	
5	Hu IL-2	58	28	46–65 (CD) [25] 65 (XR) [26]	23–25 (CD) [25]	133 [34]	4 [34]
6	Hu IL-3	54	7			133 [35]	5 [35]
7	Hu IL-4	61	31			129 [36]	
8	Hu IL-5	56	19			112 [37]	
9	Hu IL-6	67	6			184 [38]	4 [38]
10	Hu IL-7	72	16			152 [39]	
11	Hu M-CSF	55	1			165 [40] *	4 [41]
12	Hu G-CSF	73	27	66 (CD) [54]	17 (CD) [54]	177 [42]	4 [42]
13	Hu GM-CSF	55	0	47 (CD) [28]	46 (CD) [28]	127 [43]	4 [43]
14	cMGF	71	7			178 [10]	

* Explanations are given in the text.

they practically coincided with the data for IFN-αA, we shall consider the results of secondary structure predictions presented at the top of Fig. 1 (general) as common ones for IFNs-α, β, ω.

Recently the three-dimensional structure of IL-2 has been discovered [26]. A schematic model of this structure and the supposed interaction with receptors are shown in Fig. 2B. It was supposed earlier that IL-2 and IFNs must have a common, in general features, three-dimensional structure [6]. Therefore, we attempted to fold the predicted α-helical segments of IFNs in the three-dimensional structure established experimentally for IL-2 [26]. The result is shown in Fig. 2A. It should be noted that in the IL-2-like model of IFNs (Fig. 2A), cystéine residues are found at distances not hindering the formation of disulfide bridges between them. In general, the direction of the polypeptide chain in the model presented in Fig. 2A, coincides with the predicted structure shown in Fig. 6 and Fig 7A of Ref. 13. In the model of IL-2 presented in Fig. 2B, the regions important for biological activity (according to the experimental data [25,44]) are shaded.

It is known that there exists the correlation between the conservativity of amino acids at definite positions and their affiliation to structurally and/or functionally significant fragments of the polypeptide chain.

Our analysis of conservative positions in 72 IFNs reveals 17 positions at which there are no substitutions or only one exists (the alignment of 72 IFNs was kindly provided by Dr. A. Ya. Strongin, Institute of Molecular Genetics, Moscow, personal communication). These positions are indicated in Fig. 2A by circles (shaded circles denote hydrophobic amino acid residues, unshaded the hydrophilic ones). Figures near one-letter symbols correspond to their positions in the amino acid sequence of the mature protein. As seen from Fig. 2A, all the conservative residues can be divided into three clusters: the 'loop' one: L_3, L_{30}, R_{33}, F_{36} and P_{39}; 'hydrophilic': E_{59}, Q_{62}, S_{73} and Q_{92}; and 'hydrophobic': L_{96}, Y_{123}, Y_{130}, L_{131}, C_{139}, A_{140}, W_{141} and V_{144}.

Conservative residues of the 'hydrophobic' cluster are located on the converged segments of helices D and E and seem to serve for stabilization of the similar, for all IFNs, conformation of the loop between the helices. Attention to the role of the given loop for IFN function still increases due to the homology revealed between the corresponding sequence of IFN-α_2 and thymus hormone, PTM-α_1 [4]. Fig. 3 shows the alignment of the C-terminal sequence of IFN-α_2, starting from residue 116, and of PTM-α_1. Probability of random coincidence for the sequences compared is $P \approx 5 \cdot 10^{-4}$.

Probability of random coincidence was determined

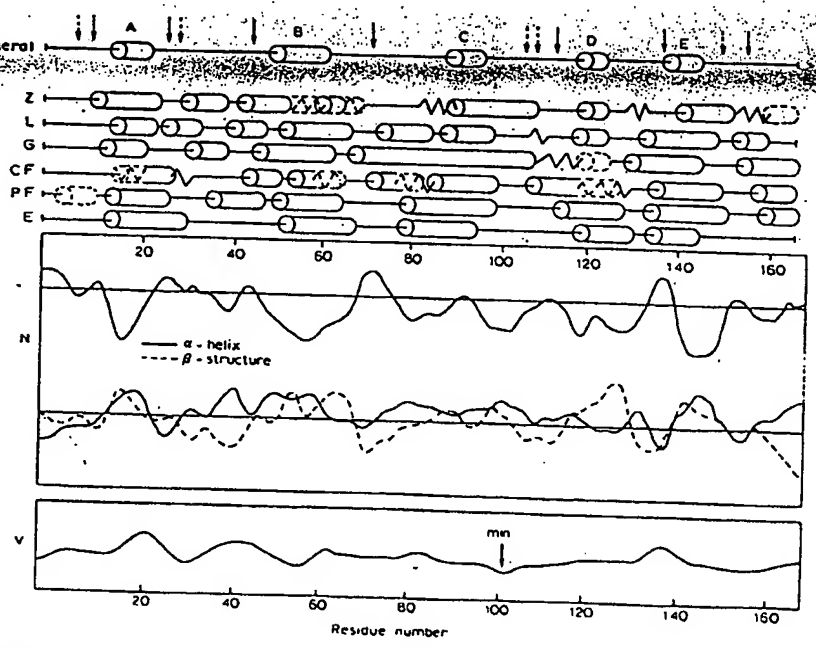

Fig. 1. The data of the analysis of IFN-αA secondary structure by the methods of Zav'yalov [15] (Z), Novotny [16] (N), Efimov [17] (E), Lim [18] (L), Ptitsyn-Finkelstein [19] (PF), Chou-Fasman [20] (CF), Garnier [21] (G) and the results of determination of domain borders by the method of Vonderviszt [23] (V). Cylinders and zig-zag lines indicate probable positions of formation of α-helical and β-pleated segments, respectively. Broken lines denote secondary structures, the assignment of which remains ambiguous (β-strands or α-helices). Solid arrows indicate the predicted positions of turns, broken arrows show the positions of proteolytic cleavage [53]. The segments of secondary structures predicted by all the methods used are designated as general.

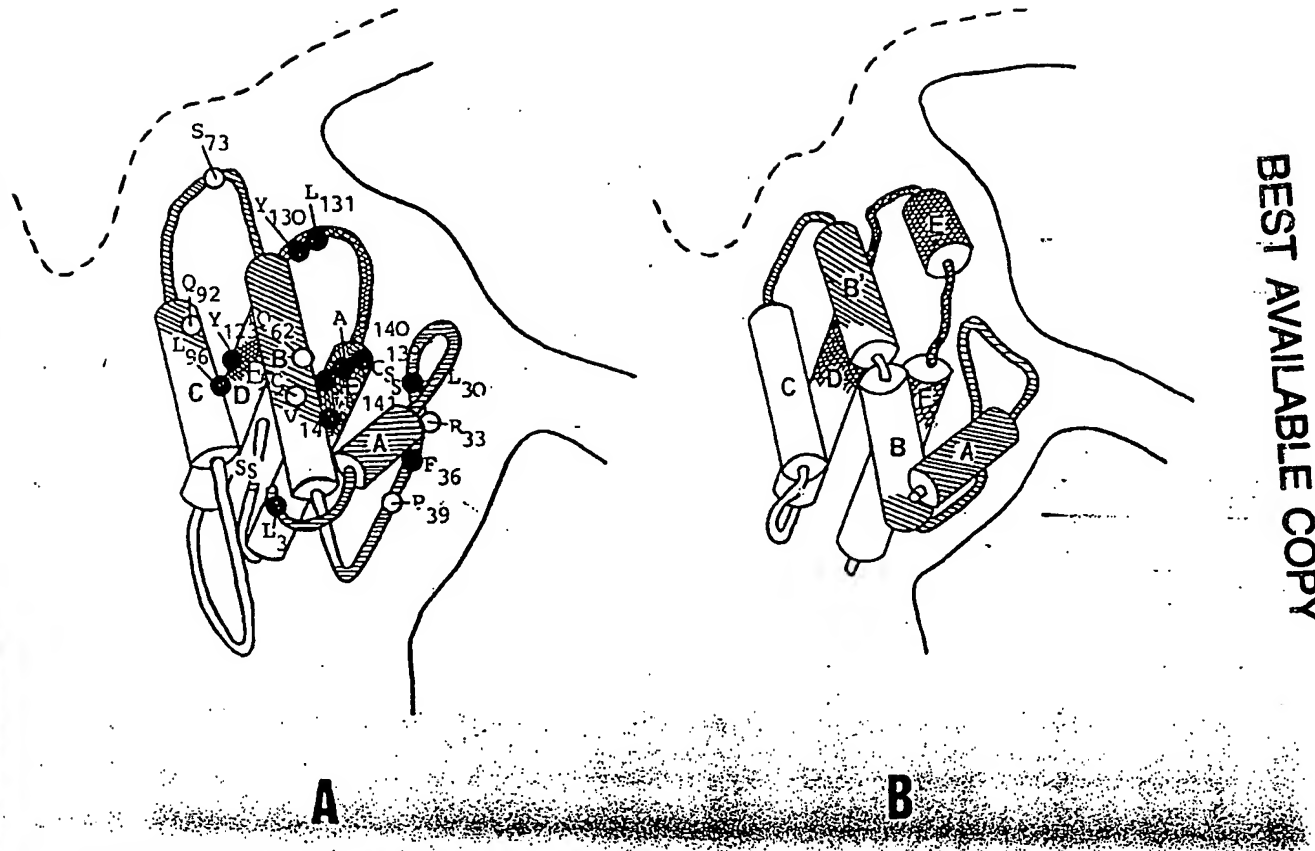

Fig. 2. (A) – Schematic presentation of the putative three-dimensional IFN structure obtained due to folding of the predicted α-helical segments of IFNs-α, β, ω into the three-dimensional structure established experimentally for IL-2 [26] – (B). The α-helical segments in accord with their sequence from the N- to C-termini of the polypeptide chain are designated by cylinders and large letters in alphabetic order. Conservative residues in IFNs-α, β, ω are indicated by circles with symbols and numbers: open circles denote hydrophilic residues, shaded circles hydrophobic residues. The letters SS mark the positions of disulfide bonds. The supposed active sites in IFNs and IL-2 are shaded. The curved line indicates the outline of the putative binding sites of receptors.

according to the formula: $P = W \cdot Z \cdot X$ [22]. The W value (probability of obtaining 'by chance' of random coincidences between two proteins in the region H amino acids long) was estimated from the binomial distribution of random values:

$$W = \sum_{I=n}^{I=H} \frac{H!}{I!(H-I)!}\left(\frac{1}{20}\right)^{I}\left(\frac{19}{20}\right)^{H-I}$$

where n is the number of coincidences. In calculations the number of possible independent comparisons between primary structure fragments including deletions or insertions must be taken into account. Z denotes the number of deletion distributions:

$$Z = \frac{(H-L-1)!}{K!(H-L-K-1)!} \cdot 2^{K}$$

where K is the deletion number and L is the summation length of deletions, X is the number of combina-

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                    120         130         140         150         160
                    *  **       *  *****       ***  **  *  *          *  **
HU IFN-ALPHA 2  SILAVRKYFQRITLYLKEKKYSPCAWEVVR-AEIMR-AE-IMRSFSLSTNL-QE
PTM    -ALPHA 1  SDAAVDTSSEITTKDLKEKK------EVVEEAENGRDAPANGNAQNEENGEQE
                         10          20          30          40
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Fig. 3. The alignment of primary structures of IFN-α_2 [29] and PTM-α_1 [52].

tions of deletion lengths:

$$X = \frac{(L-1)!}{(K-1)!(L-K)!}$$

It should be noted that the five-member fragment LKEKK identical for IFN-α_2 and PTM-α_1 corresponds to the loop between helices D and E in IFNs. Due to the fact that PTM-α_1 is an immunomodulatory hormone, it is reasonable to assume that the loop formed by helices D and E is a part of the site responsible for IFN immunomodulatory activity.

Assuming that the conservative amino acids of the 'loop' and 'hydrophilic' clusters as well as the loop between helices D and E directly participate in the formation of active sites of IFNs-α, β, ω, in Fig. 2A we indicated the orientation of the molecule relative to the tentative receptor. It is seen that the putative character of the interaction of IFNs-α, β with the receptor is similar to analogous interaction for IL-2 (Fig. 2B) [26].

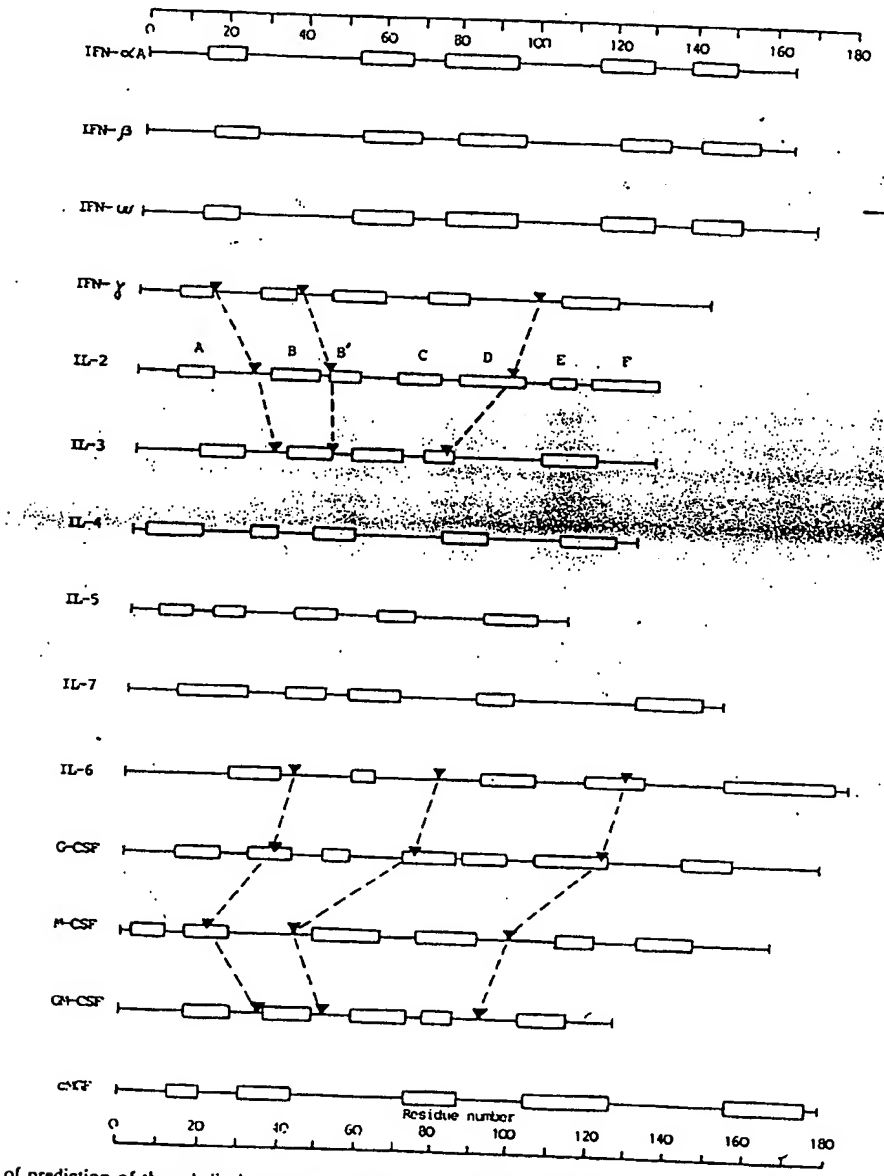

Fig. 4. The results of prediction of the α-helical segments capable of forming tightly packed hydrophobic core [17] from amino acid sequence of IFN-α, β, ω, γ; IL-2, 3, 4, 5, 6, 7; CSF-M, G, GM and cMGF. Cylinders indicate α-helical segments. Shaded triangles denote the boundaries of the segments coded by separate exons.

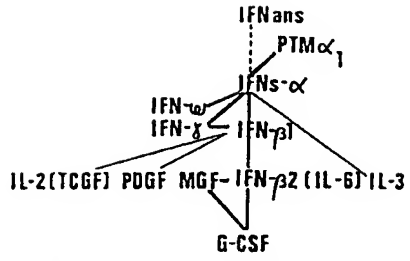

Fig. 5. The putative evolutionary relationships among proteins of the superfamily of α-helical globular (interferon-like) proteins. Double lines indicate the statistically valid homology of primary structures, solid lines the statistically valid homology of the hydrophobic cores [6,7], broken lines indicate functional relationship.

Fig. 4 shows the results of secondary structure predictions for the proteins listed in Table I. The computation was carried out by the method of Efimov [17], which determines the localization of the segment of α-helices capable of building in the hydrophobic core. The proteins are seen to have a definite extent of similarity in the secondary structure. In most cases, in a separate mature protein, five α-helical segments are identified. Besides, Fig. 4 presents the accumulative data on the intron localization along the amino acid sequence. There exists a certain correlation between the location of α-helices and that of introns. The first and second exons contain, as a rule, one helix, the third exon two helices and the fourth contains the remaining α-helical segments. The exception is IL-3 whose additional intron is located between the two last α-helices.

In addition to analogies in secondary structure and intron-exon organization of the gene, in a number of cases, the homology between the proteins under analysis is observed directly in primary structures. Thus, the homology is shown between IFN-α, β and IFN-γ [46], IFN-β and IFN-β_2 (IL-6) [45], IFN-β_2 and G-CSF [9], cMGF and G-CSF and IL-6 [10], IL-3 and IL-5 [11]. Table II presents estimations of probability of random coincidences (P) of these homologies. It can be considered that only in case of IL-3 and IL-5 the observed homology [5] is not statistically valid. Fig. 5 shows possible evolutionary relationships between these proteins. Double lines indicate the statistically valid homology in the fragments of primary structures compared. Single lines denote the statistically valid homology in the structure of hydrophobic core [6,7].

Discussion

In the present work attempt is made to analyse possible structure–evolutionary relationships between immunocytokines. The analysis of their possible secondary structures demonstrated that most of them are α-helical proteins sharing some common features of distribution of α-helices along the polypeptide chain (IL-1 and TNF were not analysed in this paper, since the X-ray analysis revealed the β-structure type of their folding [47,49]). In particular, the relationship between the location of α-helices and that of introns was demonstrated. However, these data are insufficient to deduce the existence of a common evolutionary origin and similarity in the three-dimensional structures of the proteins under study. Nevertheless, for some proteins

TABLE II

Probability of random coincidence of amino acid residues in immunocytokines

No.	Protein name	Numbers of the N- and C-termini of the fragments compared		Alignment compared H	Quantity of deletions K	Overall deletion length L	Quantity of coinciding amino acids n	Probability of random coincidence P	Alignment origin
		N-	C-						
1	IFN-αA	1	163						
	IFN-β	3	166	164	1	1	55	$7\cdot10^{-28}$	[29]
2	IFN-β	67	112						
	IFN-γ	60	104	46	1	1	14	$3\cdot10^{-6}$	[46]
3	IFN-β	33	67						
	IFN-β_2	42	77	37	3	3	11	$6\cdot10^{-2}$	[45]
4	IFN-β	136	165						
	IFN-β_2	122	151	32	2	4	9	$8\cdot10^{-2}$	[45]
5	IFN-β_2	−1	70						
	G-CSF	22	92	74	3	4	18	$3\cdot10^{-2}$	[9]
6	G-CSF	1	177						
	cMGF	1	178	182	5	9	72	$2\cdot10^{-32}$	[10]
7	IL-6	2	184						
	cMGF	1	175	183	3	8	40	$4\cdot10^{-7}$	[10]
8	IL-5	41	74						
	IL-3	−1	33	37	6	6	11	47	[11]

(IFN-α, β, γ, IL-6, G-CSF and cMGF) such a conclusion is natural. So, they are shown to have a statistically valid homology in a fragment of the amino acid sequence. Besides, for a number of proteins (IFN-α, β, IL-2, IL-3) the analogies in the structure of hydrophobic cores were revealed [6,7] that provides evidence for common features of formation of their three-dimensional structures. It has been shown for IL-2 and IFN-α, β that analogies are observed both in the tertiary structure and in arrangement of probable active sites.

As a result of the analysis of structural interrelationships between proteins of the given family, some conclusions on their origin can be drawn. So, the fact that the C-terminal part of IFN-αA has a statistically valid homology with PTM-α_1 yields an assumption that the IFN-α, β genes are products of fusion of a PTM-α_1 gene with a gene of IFN-precursor. The effect of gene fusion can be manifested in the protein domain arrangement. Two-domain organization of IFNs-α, β is evidenced by the data on domain localization obtained by means of the method of Vonderviszt and Simon [23] on the basis of the protein amino acid sequences. The results of difference adiabatic scanning microcalorimetry for the recombinant IFN-α_2 indicate the existence of two unequal domains in the molecule of this protein as well [48]. At the same time the fluorescence polarization data indicate the lack of intramolecular mobility in the molecule of IFN-α_2 [48]. On the basis of the data on homology of primary structure fragments of IFNs-α, β and IFN-γ, it was supposed in Refs. 3 and 4 that the IFN-γ gene is a product of recombination of segments of IFN-α and -β genes.

Thus, one more protein family can be identified in the immune system. Similarly to the immunoglobulin superfamily [50], the given family seems to include, along with the soluble globular proteins analysed in the present work, protein receptors, adhesion factors of lymphocytes (LFA-1) and macrophages (Mac-1), whose primary structures are homologous to those of IFNs-α, β [51]. In contrast to the immunoglobulin superfamily involving β-pleated proteins, the family of interferon-like proteins is α-helical. Nevertheless, a close structure–function relationship seems to exist between these two families. For instance, the M-CSF receptor belongs to the family of immunoglobulin-like proteins [50].

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